CODING FORMS FOR SRC INDEXING

Microfiche No.	
OTS0572595	

New Doc ID	Old Doc ID
86950000289	

Date Produced	Date Received	TSCA Section
05/10/95	07/28/95	8D

Submitting Organization
INTL ISOCYANATE INSTIT INC

Contractor
CARNEGIE MELLON UNIV

Document Title
CONJUGATE SERA BANK (CSB) PROJECT: STANDARDIZATION OF DIISOCYANATE CONJUGATE PREPARATION AND ANTISERA SCREENING WITH COVER LETTER DATED 07/25/95

Chemical Category
2,4-TOLUENE DIISOCYANATE (CAS# 584-84-9)

CODING FORM FOR GLOBAL INDEXING

Microfiche No. (7) •		1	No. of Pages	2			
Doc I.D.	3	Old Doc I.D.	8695000289				
Case No.(s)				5			
Date Produced (6)	6	Date Rec'd (6)	7	Conf. Code •	8		
				N			

Check One: ☐ Publication ☐ Internally Generated ☐ Externally Generated

Pub/Journal Name							9
							9
Author(s)							10
Organ. Name							11
Dept/Div							12
P.O. Box	13	Street No./Name					14
City	15	State	16	Zip	17	Country	18
MID No. (7)	19	D & B NO. (11)					20
Contractor							21
Doc Type • • •				8D			22
Doc Title							23

Chemical Name (300 per name)	25	CAS No. (10) 24

INTERNATIONAL ISOCYANATE INSTITUTE, INC.
119 CHERRY HILL ROAD
PARSIPPANY, NEW JERSEY 07054
TELEPHONE: (201) 263-7517
FAX: (201) 263-8739

July 25, 1995

Contains No CBI

SENT BY CERTIFIED MAIL

Attn: TSCA Section 8(d) Coordinator
Document Control Officer
Office of Pollution Prevention & Toxics (TS-790)
U.S. Environmental Protection Agency
401 M Street, S.W.
Washington, D.C. 20460

Dear Sir or Madam:

In accordance with 40 CFR 716.30, the International Isocyanate Institute (III) on behalf of its members (BASF Corporation, Bayer Corporation, Dow Chemical Company, ICI Americas, Inc., and Olin Corporation) hereby provides a copy of the following interim report as a follow-up to our study in progress notification (attached) dated February 22, 1995.

Conjugate Sera Bank (CSB) Project: Standardization of Diisocyanate Conjugate Preparation and Antisera Screening.

Very truly yours,

R. K. Rigger
Managing Director

RKR/sha
Enclosure

RECEIVED
OPPT CBIC
95 JUL 28 PM 3:17

86956000289

95 AUG 28 AM 9:08
RECEIVED
OPPT NCIC

INTERNATIONAL ISOCYANATE INSTITUTE, INC.
119 CHERRY HILL ROAD
PARSIPPANY, NEW JERSEY 07054
TELEPHONE: (201) 263-7517
FAX: (201) 263-8739

February 22, 1995

SENT BY CERTIFIED MAIL.

Attn: TSCA Section 8(d) Coordinator
Document Control Officer
Office of Pollution Prevention and Tox
US Environmental Protection Agency
401 M Street, S.W.
Washington, DC 20460

Contains No CBI

Dear Sir or Madam:

The International Isocyanate Institute (III) on behalf of it members (BASF Corporation; The Dow Chemical Company; ICI Americas Inc.; Miles, Inc.; and Olin Corporation) hereby notifies the EPA of the Conjugate Serum Bank (CSB) Project. The CSB plans to develop standardized methods for diisocyanate conjugate preparation and sera testing. We hope to establish a centralized bank of well characterized conjugates and antisera for use by clinicians and researchers. While believe that this project does not qualify as a "study" under 8d reporting requirements, we are using this 8(d) notification procedure to help to apprise EPA and the scientific community of this activity. Phase 2 plan of the CSB project begins January 1, 1995.

Name of Chemical Substance:	Toluene-2-4-diisocyanate
Chemical Abstract Service Number:	584-84-9
abbreviation:	TDI
Name of Chemical Substance:	Isocyanic Acid, Polymethylene-polyphenylene ester
Chemical Abstract Service Number:	9016-87-9
abbreviation:	polymeric MDI
Description:	Conjugate Serum Bank
Name and Address of CSB:	Dr. Amy L. Kennedy CSB Project Director Dept. of Biological Sciences Carnegie Mellon University 440 Fifth Avenue Pittsburgh, PA 15213

Very truly yours,

R.K. Rigger
Managing Director

Study Title

Conjugate Sera Bank (CSB) Project: Standardization of Diisocyanate Conjugate Preparation and Antisera Screening

Project ID: 107 AM-MTX

Contains No CBI

Author(s)

A.L. Kennedy and W.E. Brown

Interim Report Date

May, 1995

Performing Laboratory

The Laboratory of Dr. William E. Brown
Department of Biological Sciences
Carnegie Mellon University
4400 Fifth Avenue
Pittsburgh, Pennsylvania 15213
PH: (412) 268-3416
FAX: (412) 268-7129

Sponsor

International Isocyanate Institute, Inc.

SIGNATURE PAGE

Compound: **2,4-Toluene Diisocyanate**

Title: Conjugate Sera Bank (CSB) Project: Standardization of Diisocyanate Conjugate Preparation and Antisera Screening

Amy L Kennedy 5/10/95

A.L. Kennedy, Ph.D. (Date)
Project Director

5/11/95

William E. Brown, Ph.D. (Date)
Laboratory Director

Donald J. Aul 5/10/95

Donald J. Aul (Date)
Research Assistant

Timothy D Landry 5/11/95

Timothy D. Landry, Ph.D. (Date)
Task Force Chairman,
Project Monitor

TABLE OF CONTENTS

		PAGE
SIGNATURE PAGE		**2**
TABLE OF CONTENTS		**3**
LIST OF FIGURES AND TABLES		**4**
SUMMARY		**5**
INTRODUCTION		**6**
PURPOSE OF PROJECT		**9**
PROJECT DESIGN		**9**
MATERIALS AND METHODS		**11**
Chemicals	11	
Liquid Test Conjugate Generation System	11	
Vapor Conjugate Generation System	12	
Determination of Test Conjugate Protein Concentration	12	
Determination of Degree of Amino Group Modification	13	
Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel Electrophoresis	13	
Isoelectric Focusing Polyacrylamide Gel Electrophoresis	13	
Spectrophotometric Characterization	13	
Dot Blot Immunological Screening	13	
ELISA Immunological Screening	14	
RESULTS		**15**
Administrative Component		**15**
Experimental Component		**17**
Conjugate Synthesis and Characterization	17	
Immunological Screening	22	
DISCUSSION		**33**
REFERENCES		**34**

LIST OF FIGURES, TABLES AND APPENDICES

		PAGE
FIGURE 1:	Potential Mechanisms Isocyanate-Induced of Occupational Asthma	9
FIGURE 2:	*In Vitro* Vapor Modification System	18
FIGURE 3:	TNBS Reaction Time Course	20
FIGURE 4:	Spectral Analysis of TDI-HSA Test Conjugate	21
FIGURE 5:	SDS PAGE of Vapor and Liquid Phase Conjugates	23
FIGURE 6:	Assessment of Reaction Reproducibility by SDS PAGE	24
FIGURE 7:	Representative Response Profiles from CSB Serum Sample Screen	25
FIGURE 8:	CSB Serum Samples S1-S28 Screen Response	27
FIGURE 9	CSB Serum Samples S29-S46 Screen Response	28
FIGURE 10:	CSB Serum Samples S47-S76 Screen Response	29
FIGURE 11:	IgG/IgE Reaction Profiles	30

TABLE 1:	Inter-Laboratory Variations in Patient Diagnosis	6
TABLE 2:	Examples of Published Conjugate Preparation Procedures	7
TABLE 3:	CSB Project Phase I Goals and Status Summary	10
TABLE 4:	CSB Project Phase II Goals and Status Summary	10
TABLE 5:	Test Conjugate Prioritized Matrix	11
TABLE 6:	Collaborator Summary	15
TABLE 7:	Serum Sample Distribution	16
TABLE 8:	Conjugate Characterization Methods	19
TABLE 9:	ELISA Serum Titer Determinations	31
TABLE 10:	Positive Conjugate Panel Reaction Summary	32

APPENDIX I:	Medical Data Document Form	37
APPENDIX II	Conjugate Preparation Form	42
APPENDIX III:	*THE BANK* Newsletter 1.1	45

SUMMARY

Isocyanate-induced respiratory disease is one of the foremost concerns of the isocyanate industry as well as, clinicians and researchers in the field. Due to the complex and varied responses observed, difficulties have been demonstrated in accurate diagnosis, treatment and prevention. This problem is particularly evident in the analysis of the specific IgG and/or IgE antibodies. Many reports in the literature confirm this issue. At the 1992, III- sponsored, Warren House Conference, the idea of a collaborative conjugate and serum bank was developed. Protocol preparation and approval began and in August of 1993, the formal project was initiated. To date, 6 international groups have agreed to participate and 106 serum samples have been collected and tested. To the extent possible, clinical and exposure data have been collected for each sample. In parallel, 62 variable parameter conjugates have been synthesized and characterized biochemically. Immunologic screens have been performed and a panel of 6 characterized conjugates has been developed. Quality control and bulk preparation tests have also been completed. The TDI phase I of the project is completed and summarized in the following report. The MDI phase I is now in progress. The results of the initial phase confirm the importance of conjugate characterization and support the hypothesis that the high degree of variable reactivity which has been shown for numerous functional groups*in vivo* as well as for *in vitro* conjugates can affect immunorecognition.

INTRODUCTION

Occupational asthma is one of the major current health concerns (1). Isocyanate compounds have been cited as one of the most significant causes of occupational asthma which result from low molecular weight compound exposure (2). As the number of affected individuals continues to increase, clinicians and researchers are striving to understand the associated diseases and to improve diagnosis, treatment and prevention.

One of the common diagnostic tests for occupational asthma has been immunologic screening of worker sera for specific antibodies (3). In the case of isocyanates, immunologic screening methods such as ELISA or RAST have often given inconsistent results when correlated to clinical symptomology or inhalation challenge data. In some instances, even the same serum sample tested in two different laboratories can result in disparate results (Table 1).

Table 1: ELISA Screen Results for DL Sera at Two Independent Laboratories

Laboratory #1 ELISA Results (OD 405 nm)

Test Conjugate	IgG			IgE		
	1/10	1/100	+/-	1/5	1/10	+/-
MDI-HSA	.211	.125	-	.129	.125	-
HSA	.193	.119	-	.151	.138	-
Neg. Control	.168	.141	-	.133	.131	-

Laboratory #2 ELISA Results (OD 410 nm)

Test Conjugate	IgG			IgE		
	1/10	1/100	+/-	1/10	1/100	+/-
MDI-HSA	.110	.02	+	.34	.08	+
HSA	.00	.00	-	.00	.00	-
Neg. Control	.00	.00	-	.03	.01	-

With regard to specific antibody responses, a low and variable range of responder percentages have been reported (4, 5, 6, 7, 8, 9, 10) and several investigators have postulated that isocyanate-related asthma may not always be immunologically mediated (11). Clinicians are therefore left with an unreliable method of diagnosis. Some groups have totally abandoned the immunologic methods and have begun specific inhalation challenge protocols. At this time,

however, the specific challenge procedure is an expensive and difficult alternative which is not practical at the general clinic level.

Part of the problem with immunologic testing has frequently been attributed to conjugate preparation (2, 9, 12). Table 2 illustrates only a few of the published variations in isocyanate conjugate preparation methods.

TABLE 2: Examples of the Variable Parameters Used in Published Isocyanate Conjugate Preparation Procedures

Reaction Parameter	Group 1	Group 2	Group 3	Group 4	Group 5
Protein Concentration	5 mg/ml	0.5%	1%	3 mg/ml	5 mg/ml
Reaction volume	20 ml	100 ml	140 ml	20 ml	10 ml
pH		7.4 + 9.4	7.4	9.3	7.4 + 9.4
Buffer	7-9% $NaHCO_3$	50 mM phosphate 50 mM borate	PBS [a]	50 mM borate + KCl	phosphate borate
Molar Ratio	400:1	10; 50; 200	700:1	1 ml neet	25:1; 100:1
Solvent	neet	acetone	neet	neet	acetone
Reaction Temperature	RT	0 + 37°	RT	0°	RT
Reaction Time	1 hr	1-24 hr	5, 10, 20 min	20 min	1 hr
Stirring	y	y	y	y	y
Quench	n	10x molar excess mono-ethanolamine	equal vol 2 M ammonium carbonate	n	n
Post-reaction Processing	PBS	1. filter 2. dialyze 3. lyophilize	1. 5000rpm 20' 2. dialyze 3. 20% TCA 5000 xg 4. 1N NaOH	1. centricon 2. change to $NaHCO_3$	
Filtration	0.2 m	y			
TNBS assay	y	y	y	y	y
UV spectra	240-280				
Immuno-electrophor.	y	y			
Gutman assay			y		

[a] PBS : Phosphate bufferred saline

The biochemistry of diisocyanate conjugate formation has often not been carefully regarded in the preparation of test antigens for immunologic screening. Even within a single laboratory, conjugation products may be variable due to small experimental differences such as humidity, temperature and reaction time. *In vitro* reaction studies, supported by the III, have been performed in this laboratory and have shown that even minor parameter changes can result in different reaction products, and thus confirm the importance of carefully controlled reaction conditions. Parameters such as molar ratio, method of addition, delivering solvent, and reaction pH have all been shown to cause significant alterations in the conjugates produced.

For immunological monitoring tests, such as RAST and ELISA, to properly monitor antibody levels, there must be specific recognition of the test conjugate by the antibody molecule. We have hypothesized that this recognition may be quite variable due to the highly reactive nature of the isocyanate functional groups in *in vivo* systems. Immunological screening may, therefore, require multiple forms of test conjugates due to the variety of *in vivo* epitopes which may be generated, depending on the conditions of exposure. Most laboratories use a single conjugate to screen worker samples through either the RAST or ELISA methodologies. Negative results may only suggest that the appropriate synthetic epitope was not present in the particular conjugate used. False negatives as well as false positives are common to both methods for this and other reasons. This project was designed to address such issues at the molecular level. One hypothesized solution is the development of a stringently characterized, conjugate panel to replace a single conjugate system.

Low numbers of positive antibody responders and inconsistent results between laboratories can be explained, only in part, by the conjugate preparation problem. Other confounding issues can be the treatment of test sera and the methods used for antibody detection and characterization. Again, problems arise when individual groups differ in their approach. As discussed at the 1992 Warren House workshop on isocyanate sensitization, the problem of isocyanate-induced respiratory disease is complex in itself, therefore, it was seen as advantageous for researchers and clinicians working in the field to join together to minimize complicating factors and to maintain

consistency in methods. The general goal of the proposed project was to implement this cooperat' by working together to establish and standardize optimal methods for preparation and testing of diisocyanate conjugates and antisera.

PURPOSE OF PROJECT

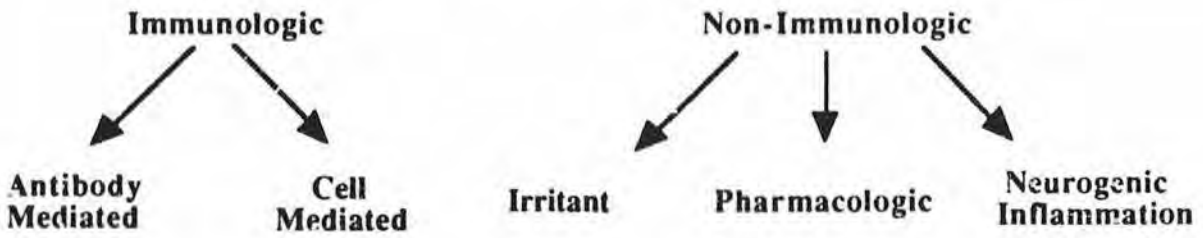
The objective of this project is to establish and standardize methods for diisocyanate conjugate preparation and sera testing. An important goal of such a project is to establish a centralized source of well characterized and tested conjugates and antisera which would be available for use by other research and clinical laboratories.

PROJECT DESIGN

There are many potential mechanisms which may be involved in isocyanate-induced occupational asthma. Figure 1 gives a brief schematic of some of these possible mechanisms.

Figure 1: Potential Mechanisms of Isocyanate-Induced Occupational Asthma

(from Asthma in the Workplace)



Despite the many research areas which could be addressed by a collaborative project on this topic, the CSB project was initially set up with a primary focus only on antibody-mediated mechanisms. Due to the extensive information which has been obtained through studies on TDI, it was decided that 2,4 TDI would be the most appropriate starting compound and that additional compounds could be included for study at later stages. Due to the known enhancement of the 2,6

isomer as foam production proceeds, many workers may be preferentially exposed to this form and 2,6 TDI conjugates will be tested to a limited extent.

The overall project was designed in phases. Due to the unique nature of the project, all phases require administrative and research components which run in parallel. This interim report is intended to summarize the current progress on the project with specific reference to the phase goals summarized in Tables 3 and 4.

TABLE 3: CSB Phase I

Specific Goal #	Goal	Progress
1	Establish collaborators	*6 labs in project *See Interaction Table
2	Design questionnaires	*Questionnaires approved and in use
3	Establish organizational routines	*Newsletter completed and distributed
4	Collect sera	* 106 sera collected *See Sera Inventory
5	Conjugate prep and characterization	*62 made and characterized
6	Screen conjugate panel with sera	*Primary screens completed *See Data Graph
7	Characterize 5-10 conjugates in detail	*In progress

TABLE 4: CSB Phase II

Specific Goal #	Goal	Progress
1	Large scale conjugate production	*Completed for 2 conjugates of 6 in panel
2	Standardize characterization	*Reproducibility tested and confirmed
3	Expand collaborator base and scientific community intact	*Grammer group invited and accepted *Meeting to coincide with ATS
4	Begin MDI start of Phase I	*Initial conjugates being prepared *MDI sera requested

MATERIALS AND METHODS

Chemicals. Human Serum Albumin (HSA) was purchased as a dried powder from Sigma Chemical CO. (St. Louis, MO). The globulin and fatty acid free fraction V form was used without further purifications. 2,4 TDI was purchased from Fluka Chemical Co. and was stored under nitrogen. Peroxidase labeled, goat anti-human immunoglobulins were purchased from ICN. Acetone and acetonitrile were HPLC grade (J.T. Baker). All other chemicals were reagent grade and were purchased from local suppliers.

Liquid Test Conjugate Generation System. Liquid phase TDI additions were performed using calibrated glass micropipettes. Delivery of TDI to a stirring protein solution was done either as a neet liquid or an acetone solution. Time between acetone dilution and delivery to the protein solution was minimized and always measured and recorded. Variable parameter reactions were performed as outlined in the prioritized matrix given in Table 5.

TABLE 5: CSB Test Conjugate Prioritized Matrix

Conjugate Parameter	Specific Condition	Number of Conjugates
pH	pH 2.3	0
	pH 7.4	36
	pH 10	23
Protein Conc.	1 mg/ml	0
	5 mg/ml	60
	10 mg/ml	2
Mode of Addition	neet liquid	3
	liquid in solvent	53
	vapor	6
Vapor Conc.	2 ppm	2
	0.2 ppm	2
	0.02 ppm	2
Molar Ratio	other	23
	2.5:1	8
	25:1	12
	100:1	6
Time of Reaction	1 hr	V:6 - L:41
	8 hr	0
	24 hr	L:12
Isocyanate Isomer	2,4 TDI	62
	2,6 TDI	0

Following the reaction period, the modified protein solutions were either subjected to filtration, precipitation, or reagent quench and dialysis.

Vapor Conjugate Generation System. For some test conjugates, vapor phase addition of the TDI was performed. A continuous airflow system was used for all TDI vapor reactions. A 2 L glass chamber was used to hold the tissue culture plate filled with the appropriate protein solution (Figure 2). To generate the TDI vapor, house air was dried, filtered and delivered over the liquid in a mini impinger with a needle. Air flow was controlled by an appropriate flowmeter and delivery rates varied between 100 ml/min and 2 L/min, depending on the target concentration. A 20 gauge needle also penetrated the top of the vial to deliver the vapor to the exposure chamber. The vapor was drawn into the system by a vacuum pump equipped with a valve and an additional flowmeter to regulate the exhaust airflow from the exposure chamber. Exhaust rates varied between 10 and 20 liters/min depending on the isocyanate concentration desired. The quantitation of isocyanate vapor concentrations in the system was performed throughout the 1 hr exposures by the periodic sampling of the chamber atmosphere using the derivatization of the reactive isocyanate with p-nitrobenzoylpropylamine (PNBPA: Regis Chemical Co.) (13) which was immobilized on glass fiber filters. Air samples were drawn through the coated glass fiber filter cassettes at a rate of 2 liters per minute for periods of 5-15 min. The filters were extracted in HPLC grade acetonitrile and an aliquot of the filter extract was analyzed by reversed phase HPLC and the area of the derivative peak was quantitated relative to a calibration curve.

Determination of Test Conjugate Protein Concentration. The protein concentration of the test conjugate solution was determined by the method of Lowry (14). Concentrations were adjusted by dilution or limited evaporation on a speed vac system. Unmodified human serum albumin samples were used to generate the calibration curve.

Determination of Degree of Amino Group Modification. The determination of free amino group concentrations was performed by the trinitrobenzosulfonic acid method of Snyder and Sobocinski (15). Unmodified HSA was used for comparison to test conjugate values and percent of amine modification was calculated.

Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel Electrophoresis. Aliquots of the test conjugates were subjected to gel electrophoresis following the procedure of Laemmli (16) using a 10% acrylamide resolving gel. The gels were stained with Coomassie blue and destained in an acetic acid/ isopropanol/ water solution. Unmodified HSA and Sigma pre-stained protein molecular weight markers were used for relative molecular weight determination.

Isoelectric Focusing Polyacrylamide Gel Electrophoresis. Aliquots of the test conjugates were also subjected to isoelectric focusing under denaturing conditions following the procedure of Robertson and co-workers (17). The gels were fixed with trichloroacetic acid (TCA), stained with Coomassie blue in methanol/acetic acid and destained in an acetic acid/ isopropanol/ water solution. Unmodified HSA and Sigma isoelectric protein standards were used for relative isoelectric point determination.

Spectrophotometric Characterization. In order to evaluate the spectral properties of the test conjugates, UV spectral scans over the wavelength range from 310 to 190 nm were performed on a sample of each conjugate using a Perkin Elmer UV-VIS spectrophotometer coupled to an IBM AT equipped with a MultiPurpose Lab Interface (Vernier). Direct scans and difference spectra relative to unmodified HSA were characterized.

Dot Blot Immunological Screening. Aliquots of the test conjugates were spotted on either nitrocellulose or nylon discs (S&S). The discs were then allowed to air dry, blocked with a 1 % nonfat dried milk solution and then blotted following a standard immunoblotting procedure (18).

CSB serum samples were used as the primary antisera and universal precautions were followed. All waste was treated as biohazardous. A commercial peroxidase-linked, goat anti-human immunoglobulin antisera was used for localization. A peroxide substrate was used.

ELISA Immunological Screening. Aliquots of the test conjugates were placed in multiwell plates and incubated at 37°C for 1 hr and then incubated at 4°C overnight. Serial dilutions of the human serum samples were performed and the plate development was done according to the procedure of Engvall and Perlman (19). Qualitative ranking was done relative to internal standards with scores ranging from 0-5. Quantitation of responses was also performed using an AMBIS 4000 scanning image system.

RESULTS

Administrative Component :

Invitation letters were drafted, approved and sent to the seven, III-designated groups. Selection of invited groups was primarily based upon involvement in the inception of the project idea at the 1992 Warren House Conference. The six laboratories that agreed to join the collaborative project are listed in Table 6. Five of these groups have supplied serum samples from TDI-exposed individuals.

TABLE 6: CSB Participant Interaction Summary

Participant Group	Response to Initial Invitation	# TDI Sera Supplied
Baur	+	4
Bernstein	+	1
Karol	+	0
Kochman	+	30
Malo	+	18 (+30 Ctrl)
Mapp/Fabbri	+	34

A total of 106 serum samples have been received to date. For the category distributions refer to Table 7. A request for serum samples from MDI-exposed individuals has been made but none have been received.

Table 7: CSB Serum Distribution

Serum Category	Total # of Samples
TDI-Exposed Workers + Asthma Diagnosis	76
TDI-Exposed Workers - Asthma Diagnosis	10
No known TDI Exposure + Asthma Diagnosis	10
No known TDI Exposure - Asthma Diagnosis	10

Dr. Marcia Lee (Dow) and Dr. Athena Jolly (ICI) designed the medical data document (Appendix I) which was also reviewed by Prof. Werner Diller. This document has been implemented and as much information as possible has been provided for the serum samples received. Correlation of clinical and exposure data with experimental results is in progress. A conjugate preparation questionnaire has also been prepared and reviewed (Appendix II).

Communication with participant groups has been ongoing. A regular newsletter was instituted and the first edition was distributed in September, 1994 (Appendix III). The second edition will be prepared for a March distribution. Plans for a CSB group meeting are being made to coincide with a professional meeting that will include occupational asthma presentations. At the suggestion of Dr. J.L Malo and Dr. Athena Jolly, Dr. Leslie Grammer (Northwestern University; Chicago, IL) was extended an invitation and has agreed to be a project participant. Expansion of the collaborator base and interaction with the scientific community was one of the goals of Phase II.

Due to the unique nature of the CSB project, regular communication with the monitoring Task Force is also crucial . Three CSB Task Force meetings have been held to review project status and Task Force conference calls have been instituted on a biweekly schedule.

Experimental Component:

Conjugate Synthesis and Characterization

The experimental phase of the project began with the synthesis of a panel of variable parameter, 2,4-TDI-human serum albumin (HSA) conjugates. A total of 62 TDI -protein conjugates have been made and characterized to date. Many of the parameters tested were based on other published methods as well as indications we had obtained from the results of our *in vitro* reaction studies on TDI. One of the most unusual parameters we have implemented is the use of a vapor phase conjugate generation system. In our previous studies with guinea pig serum albumin conjugates, we found that the mode of addition (liquid neet, acetone or vapor) significantly affected the reaction products that resulted. In addition, dot blot analysis indicated that the vapor phase conjugate had a higher level of reaction when tested with serum from inhalation-sensitized animals. We also know that route of exposure to TDI can effect the *in vivo* reaction products as well, as demonstrated by the biochemical characterization of plasma and urine from gavage and inhalation exposed rats. One of the hypotheses indirectly tested with this project therefore, was to see if this route exposure correlation to conjugate type was evidenced.

The vapor generation system used was the one designed for our *in vitro* studies and is diagrammed in Figure 2. It is modeled from the in vivo exposure systems of Alarie and co-workers. Results of our tests with ^{14}C-TDI in this system showed that there was not a positional effect in that each well received an equivalent exposure as determined by the amount of radioactivity in each well.

For each CSB test conjugate prepared, whether through liquid or vapor modification, a series of characterization methods has been implemented. These methods as well as the experimental endpoints are summarized in Table 8. These tests have also been used to assay reproducibility of conjugation methods and quality control for large scale production.

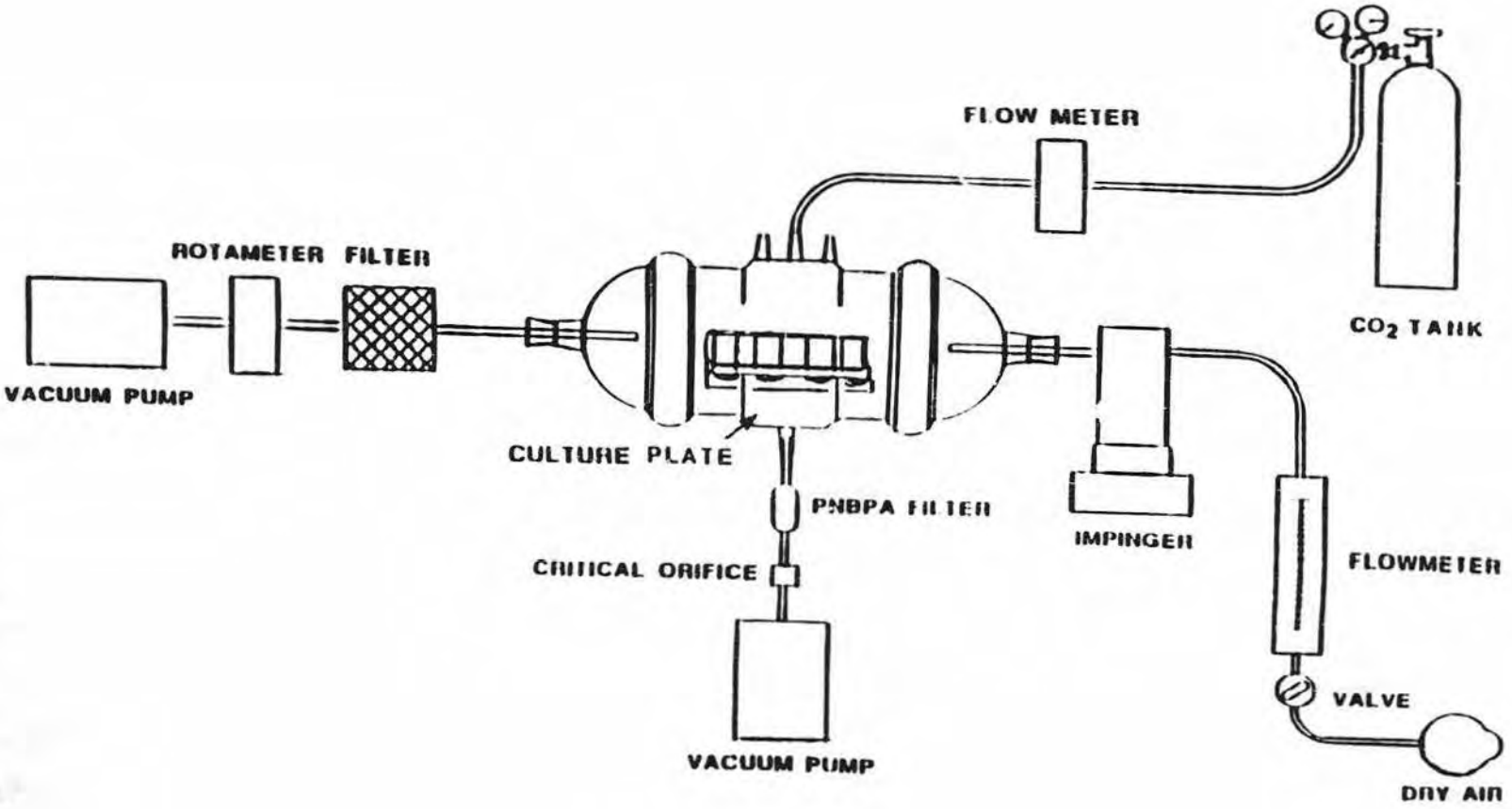

FLOW METER
ROTAMETER
FILTER
CO_2 TANK
VACUUM PUMP
CULTURE PLATE
PNBPA FILTER
IMPINGER
FLOWMETER
CRITICAL ORIFICE
VALVE
VACUUM PUMP
DRY AIR

FIGURE 2

TABLE 8: CSB Conjugate Biochemical Characterizations

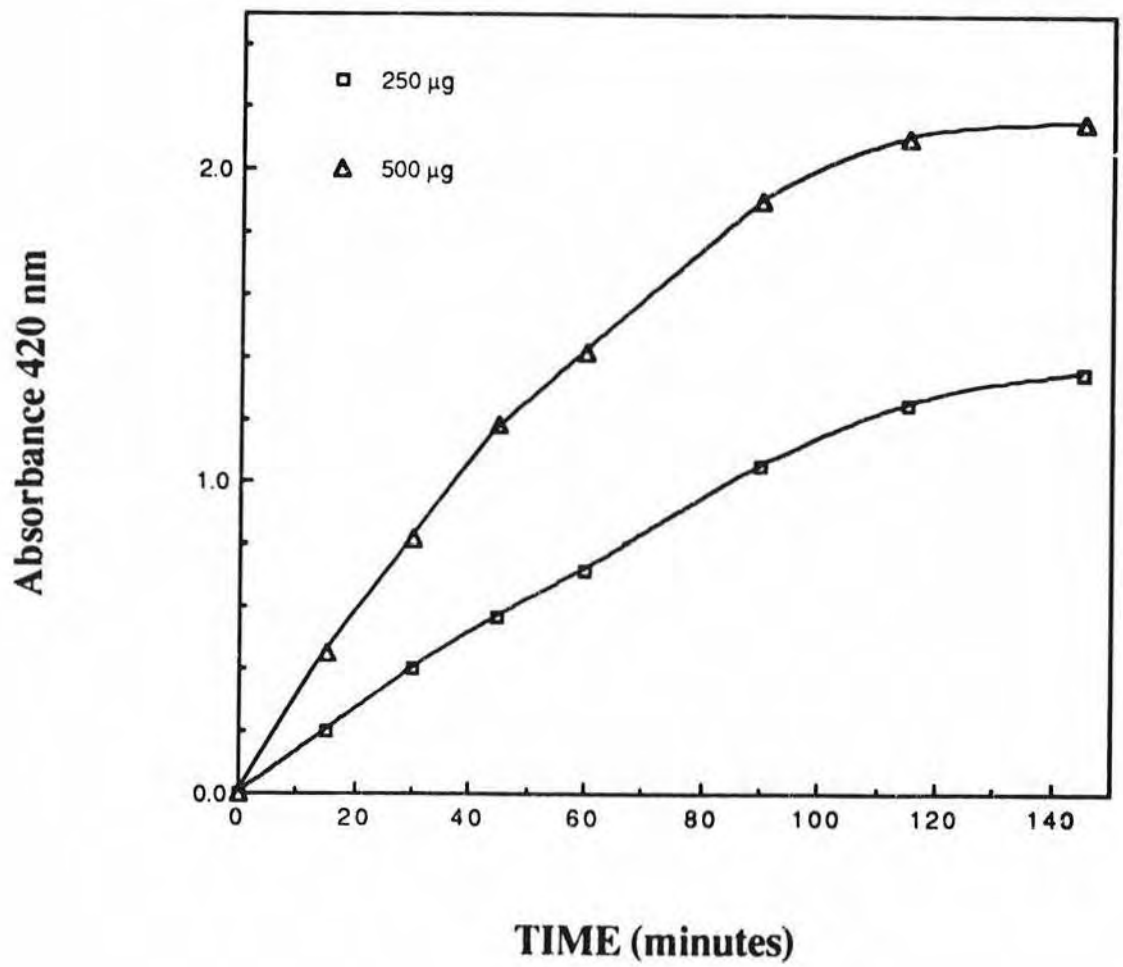
Characterization Method	Experimental Endpoint
Lowry Assay	Protein Concentration
TNBS Assay	% Amine Modification
SDS PAGE	Molecular size and shape
IEF Gel Electrophoresis	Isoelectric Point
Spectral Analysis	Chromophore Quantitation

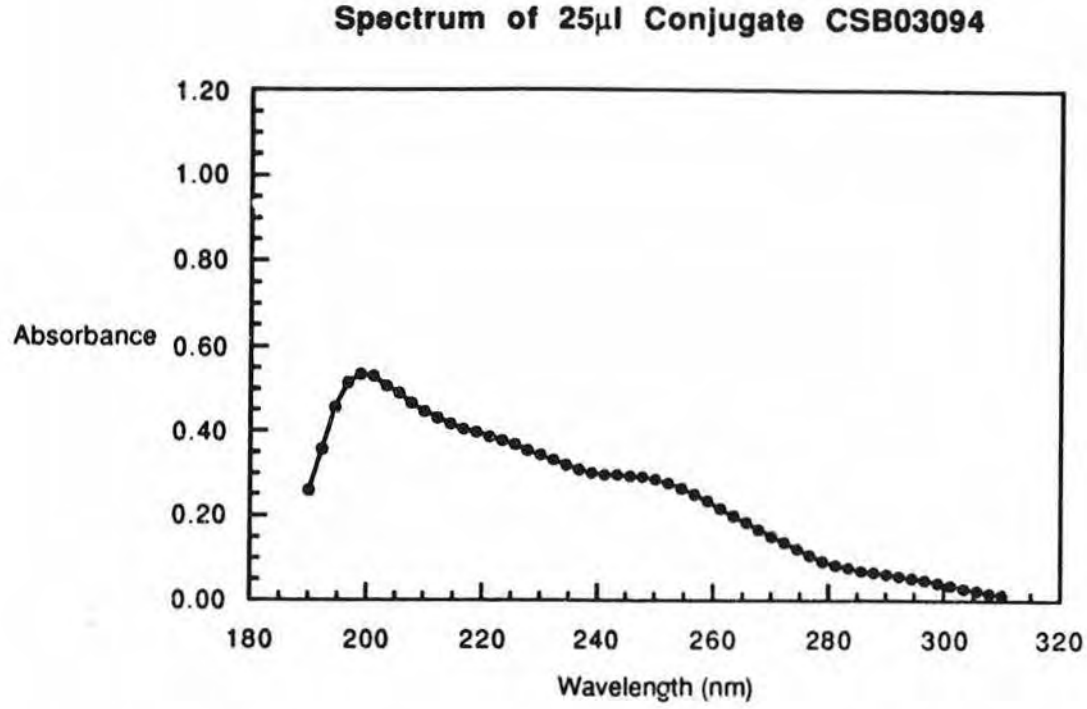
Lowry assays were run on all samples to determine protein concentrations of the final reaction product. These values were then used to standardize protein levels for use in each of the various assays. At an adjusted standard concentration (approx. 2.5 mg/ml), the conjugates were then further characterized.

The most common method of conjugate characterization found in the literature is that of TNBS amino group modification analysis. In many studies, in fact, this is the only method of characterization performed. There are however, many drawbacks with using this single approach to determine the percentage of modification. One of the major assumptions is that only amino groups will be the sites of modification. We have found that in some cases the modification percentage actually increases relative to unmodified HSA. We have hypothesized that this may be explained by mono-functional modification of a non-amino moiety on the protein with subsequent hydrolysis of the other NCO group to an amine. This would thus result in a net increase in amino groups detected. Another limitation of the method is that it assumes that the assay reaction will be operational at similar levels and rates, independent of protein structural modifications. We have done some preliminary kinetic studies with the CSB test conjugates which have shown that the TNBS reaction is much slower with the conjugates relative to unmodified HSA and that at the normal thirty minute timepoint, the absorbance values is only half the maximum (Figure 3).

We have therefore included in our conjugate characterization a number of additional methods. For example, spectral scans of the conjugates have been recorded as given in Figure 4 A. Difference spectra are then produced relative to the unmodified protein standard and a conjugate

FIGURE 3: TNBS time course of CSB 05594




Spectrum of 25µl Conjugate CSB03094
1.20
1.00
0.80
Absorbance
0.60
0.40
0.20
0.00
180
200
220
240
260
280
300
320
Wavelength (nm)

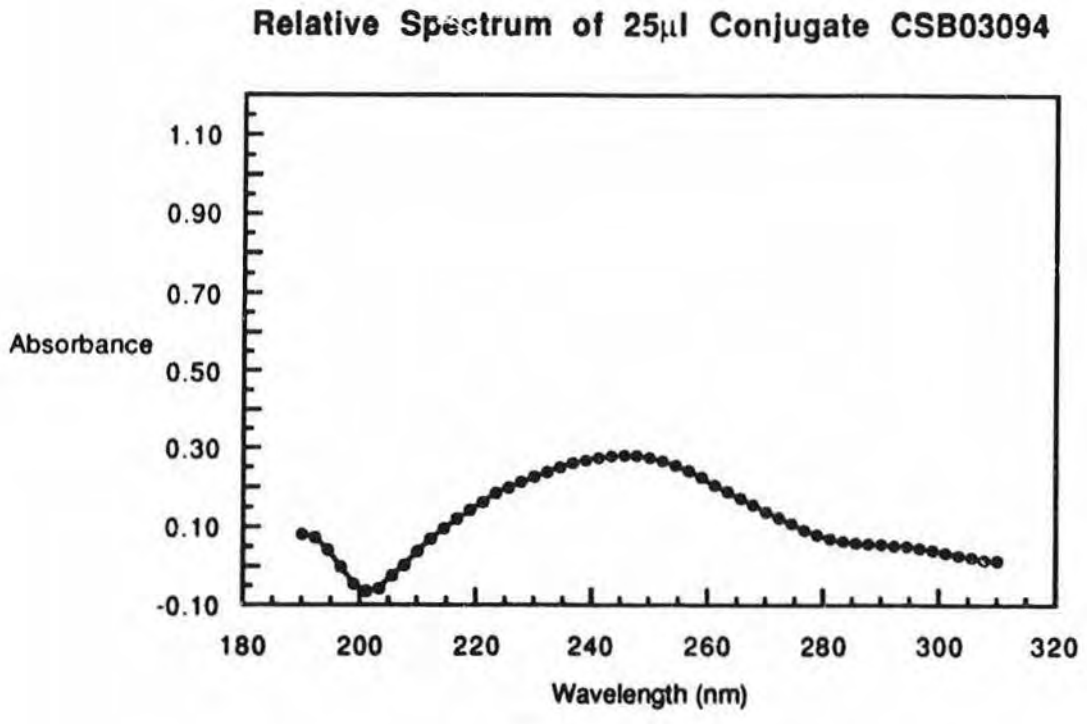

Relative Spectrum of 25µl Conjugate CSB03094
1.10
0.90
0.70
Absorbance
0.50
0.30
0.10
-0.10
180
200
220
240
260
280
300
320
Wavelength (nm)

FIGURE 4

absorbance "signature" is obtained (Figure 4B). This method is not exceptionally sensitive and therefore is to contribute to the product characterization.

One of the most striking differences observed between the individual conjugates has been their SDS PAGE electrophoretic mobility patterns. This mobility is dependent primarily on their size but structural change effects can also be seen. Protein size can be altered through bifunctional, covalent inter-molecular reactions between two or more separate protein molecules thus yielding, dimers, trimers and polymers. Structural changes are usually a result of intra-molecular covalent reactions which yield a more compact protein moiety with greater gel mobility. Examples of both of these types of reactions have been observed through gel mobility shifts, primarily for the liquid-modified test conjugates (Figure 5). As seen in our earlier studies, the vapor modified samples seem to maintain the original protein's gel mobility as shown for the unmodified albumin in lane 2 of the gel.

Differences in test conjugates have also been observed through isoelectric focusing electrophoresis. This analysis is based on the assumption that modification of the protein's functional groups at different sites and variable levels would have an impact on the overall charge of the conjugate molecule. These collective electrophoretic mobility patterns then become another "signature" of the test conjugate reaction product. When testing the reproducibility of the test conjugate preparations, this was found to be a reliable indication that the products were similar, as shown for 2 conjugates in Figure 6.

Immunological Screening

Once the group of test conjugates was prepared and characterized it was then necessary to test for immunorecognition. Serum samples from TDI-exposed workers were acquired from a number of collaborating laboratories but in very limited quantities. The screening was therefore initially performed through the use of a dot blot procedure to enable the testing of a large number of conjugates and to minimize use of protein conjugate and more importantly, the serum samples. Several strongly positive conjugates were found in the initial screens. This panel was then tested and scored for reactivity with all of the CSB serum samples. The scoring has been done both

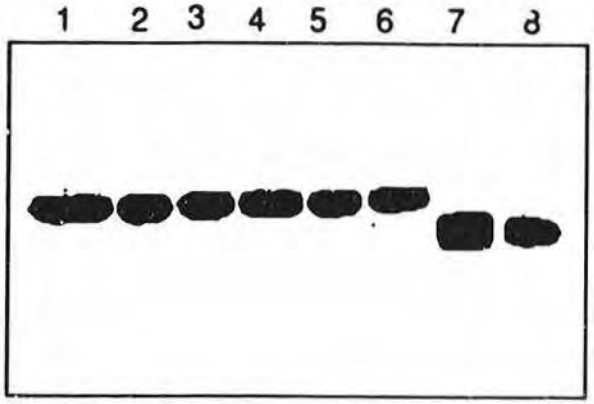


Figure 5. SDS PAGE of Vapor and Liquid Phase Conjugates

Lane Number	Sample
1	HSA pH 9.4
2	CSB Conjugate 57
3	CSB Conjugate 58
4	HSA pH 7.4
5	CSB Conjugate 59
6	CSB Conjugate 60
7	CSB Conjugate 30
8	CSB Conjugate 54

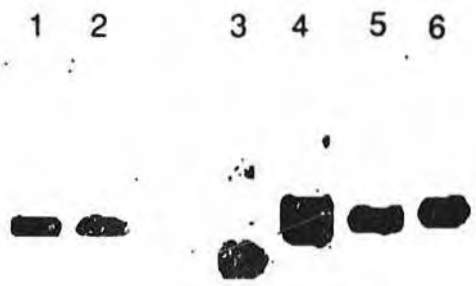
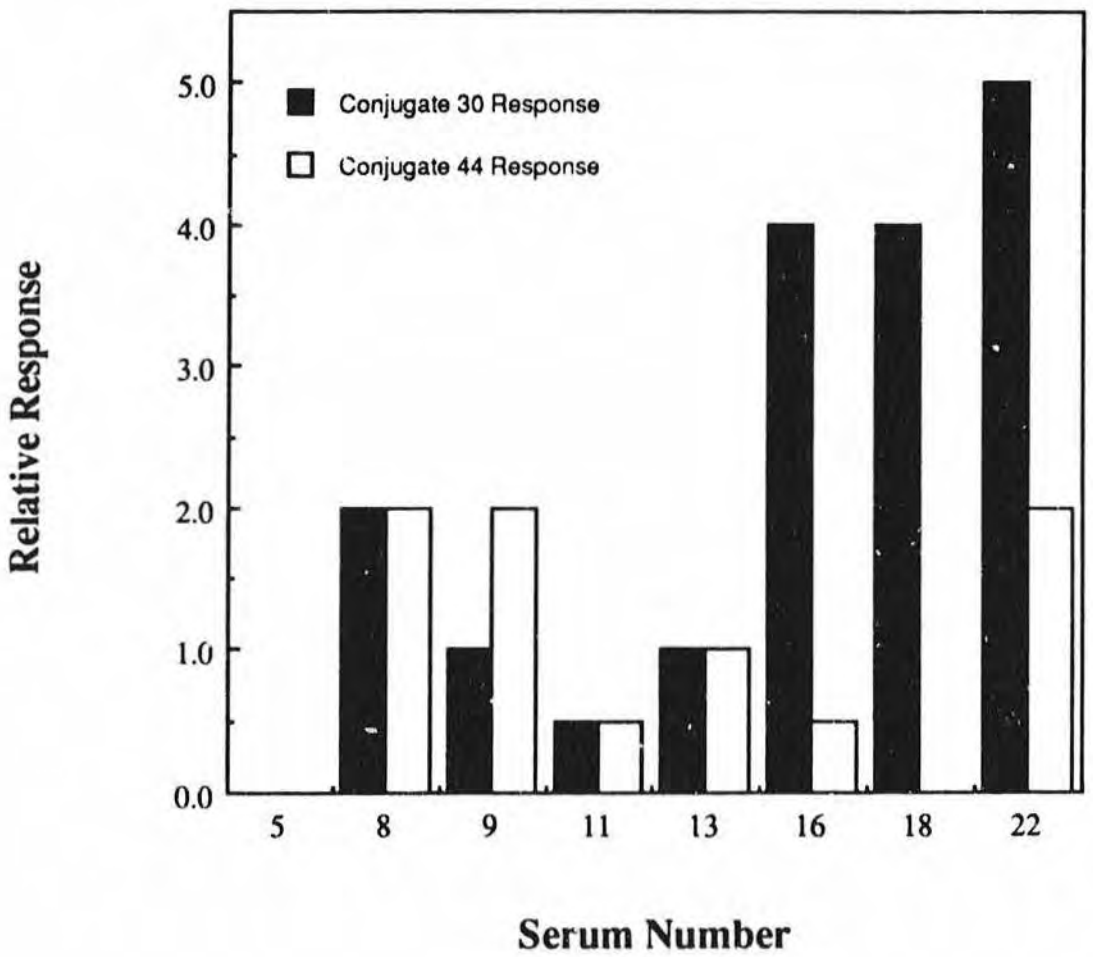


Figure 6. Assessment of Reaction Reproducibility by SDS PAGE

Lane Number	Sample
1	Control HSA, pH 7.4
2	Control HSA, pH 9.4
3	CSB conjugate 53
4	CSB conjugate 56
5	CSB conjugate 61
6	CSB conjugate 62

FIGURE 7: Representative Response Profiles CSB Sera Screen



qualitatively with an internally standardized scale and quantitatively using an AMBIS 4000 image analysis. Figure 7 provides a summary of some of the typical response profiles recorded for two of the most highly reactive conjugates. For the two different conjugates, a variety of responses was observed for a single serum sample, ranging from no detectable antibody to the highest relative value. In addition, for a single conjugate, a range of responses was also observed when numerous serum samples were tested with it. These results are of particular significance in relation to the current testing status since, most laboratories only use one conjugate. Individual screening results are given in Figures 8, 9 and 10. Based on the medical data documents which have been received for each of the serum samples, the samples with the most complete documentation have been chosen for further testing. IgG and IgE response levels are currently being assessed for this test group of 18 serum sampies. The IgG responsiveness is greater in all of the samples analyzed thus far. A representative pattern is given in Figure 11. ELISA titers are also being completed on the sera group and as shown in Table 9, results correlate well to the prior qualitative and quantitative scores.

Based on all of the screening results, a panel of test conjugates was established and includes 6 of the most highly reactive conjugates. The preparation reaction summary for the conjugates panel is given in Table 10. There are numerous differences in the reaction conditions and biochemical characterization at the levels tested thus far, there is not an apparent, common characteristic to explain their high level of immunorecognition. Detailed biophysical and biochemical characterization of these 6 conjugates is in progress and will hopefully yield a further explanation. Bulk preparations of the conjugate panel have been started and quality control assessments have been implemented and checked.

As indicated in Table 7 above, we did obtain control serum samples to test the conjugate panel and to assure the specificity of the reactions. All thirty control serum samples were screened blindly. No conjugate reaction was observed for any of the control samples. An interesting result, however is that 12 of the control serum samples showed a positive reaction with unmodified human serum albumin.

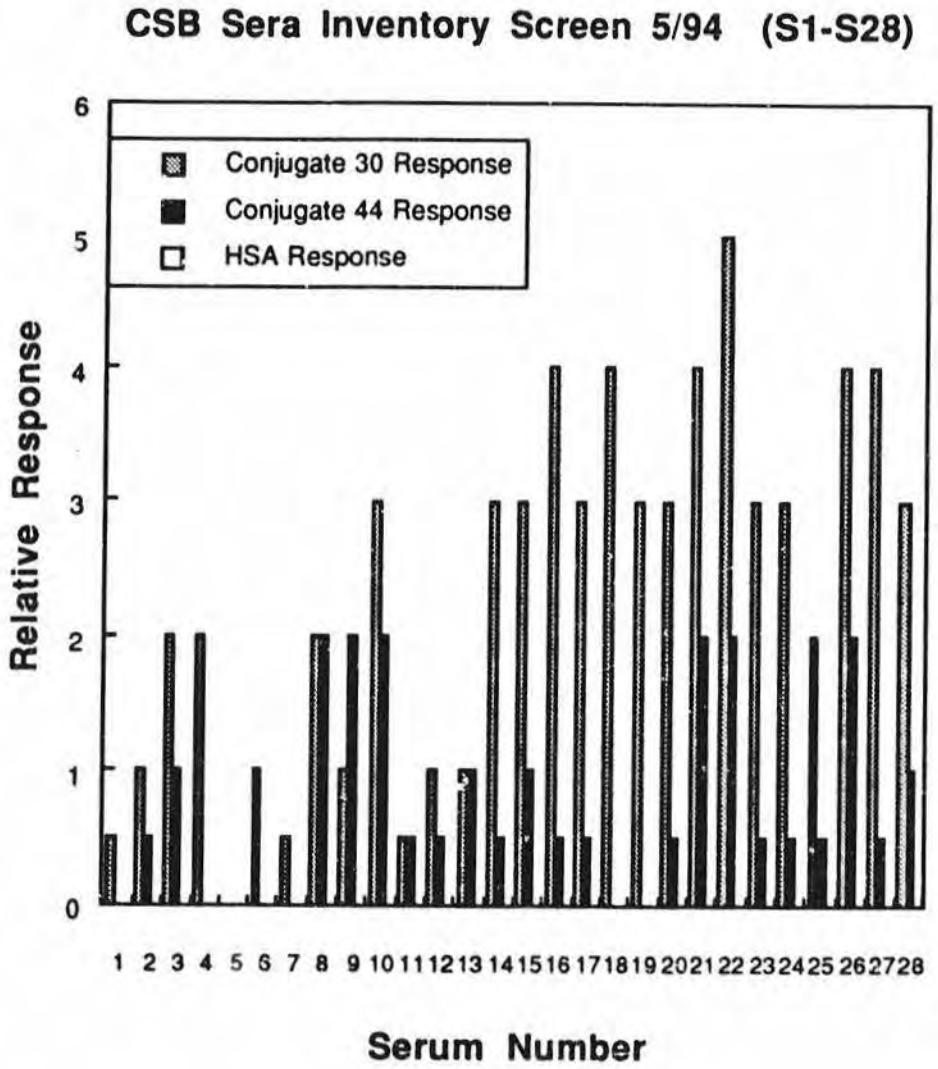

CSB Sera Inventory Screen 5/94 (S1-S28)
Conjugate 30 Response
Conjugate 44 Response
HSA Response
Relative Response
0
1
2
3
4
5
6
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
Serum Number

FIGURE 8

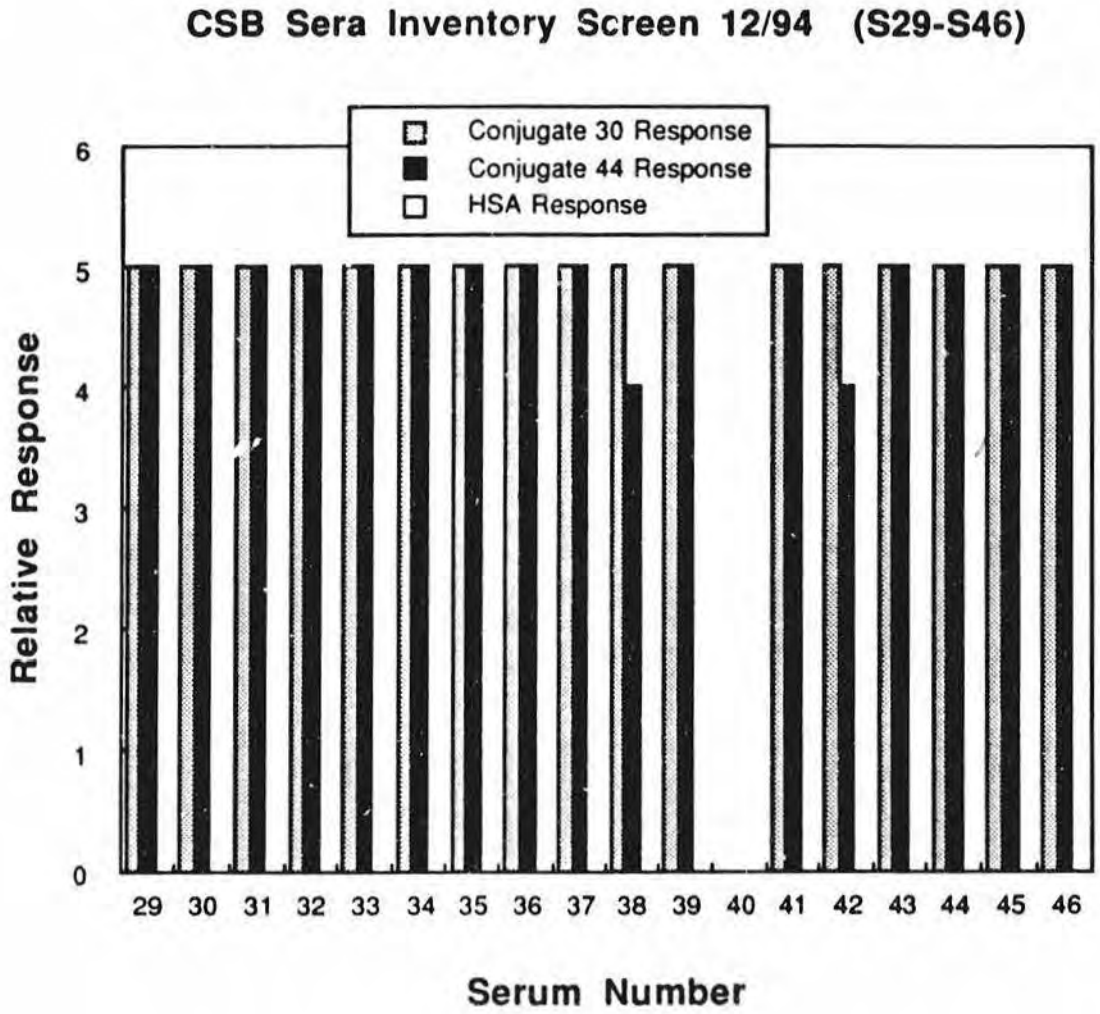

CSB Sera Inventory Screen 12/94 (S29-S46)
Conjugate 30 Response
Conjugate 44 Response
HSA Response
Relative Response
0
1
2
3
4
5
6
29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46
Serum Number

FIGURE 9

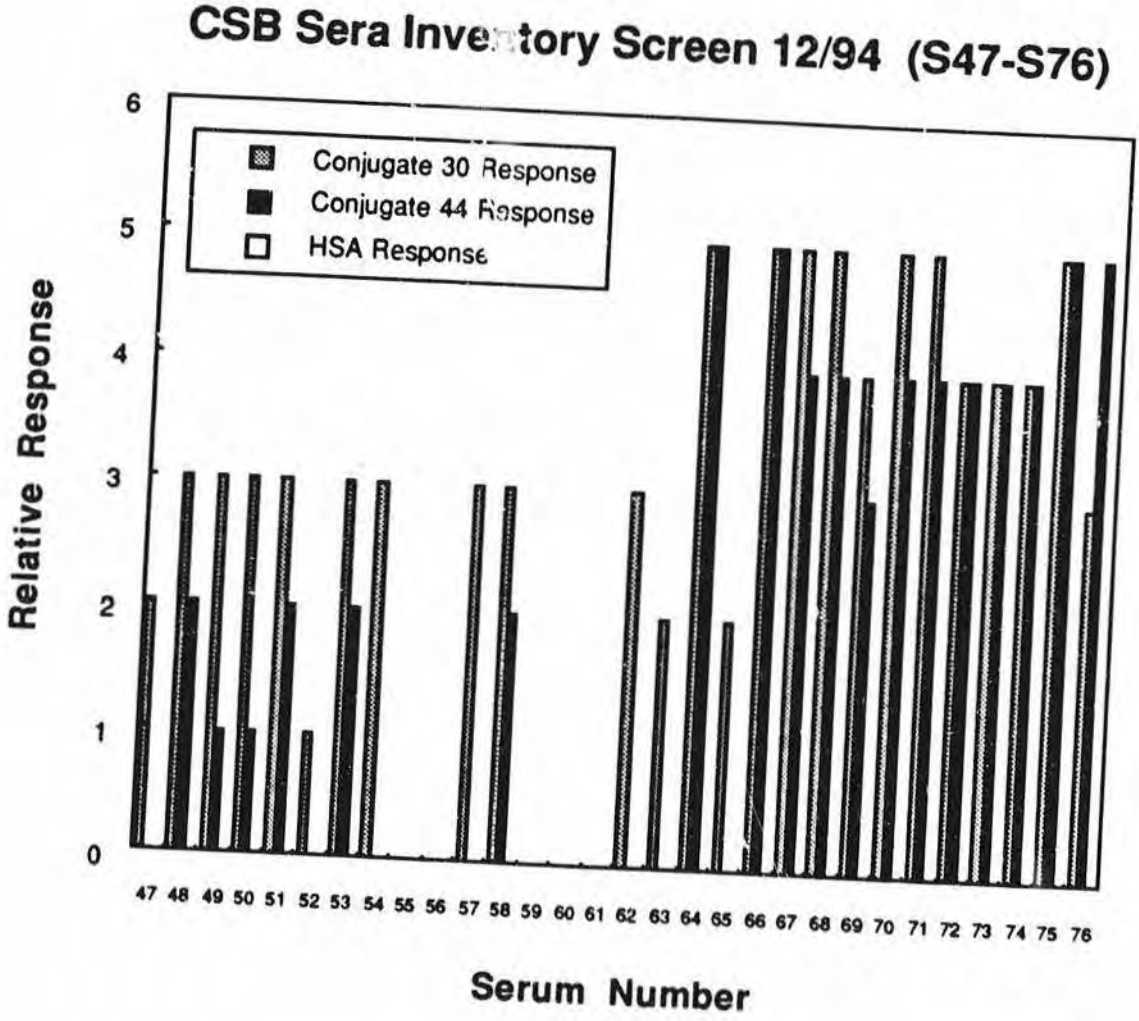

CSB Sera Inventory Screen 12/94 (S47-S76)
Conjugate 30 Response
Conjugate 44 Response
HSA Response
Relative Response
0
1
2
3
4
5
6
47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76
Serum Number

FIGURE 10

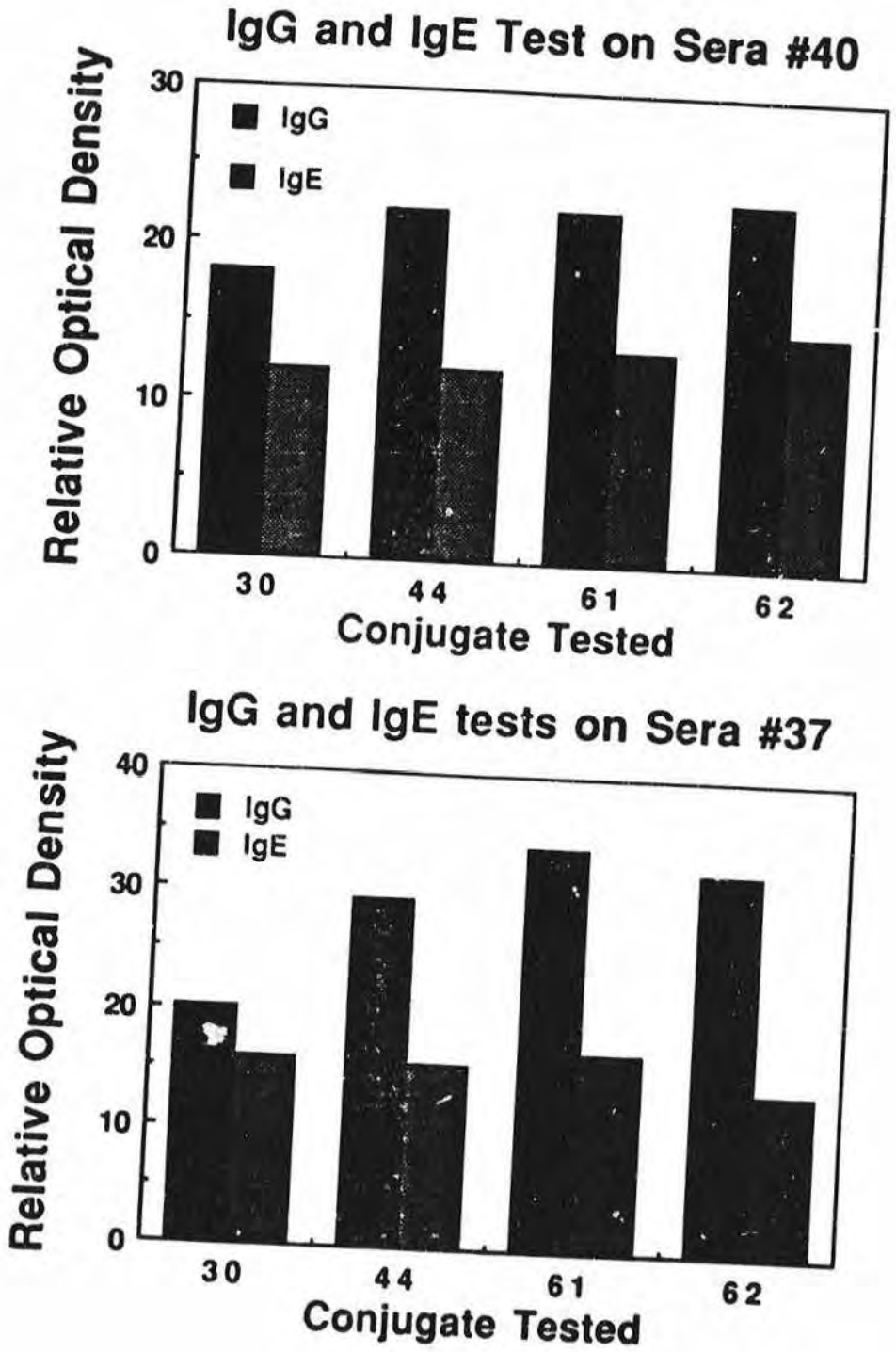

IgG and IgE Test on Sera #40
Relative Optical Density
30
20
10
0
IgG
IgE
30
44
61
62
Conjugate Tested
IgG and IgE tests on Sera #37
Relative Optical Density
40
30
20
10
0
IgG
IgE
30
44
61
62
Conjugate Tested

FIGURE 11

TABLE 9: ELISA Test on Sample Conjugates and Sera

Conjugate #	Test Sera	Titer
30	S37	2^5
30	S18	2^3
30	S8	2^2
44	S37	$>2^6$
44	S18	2^5
44	S8	2^5

Once the control distribution is released, we may be able to group and correlate this response. At any rate, non-specific reactions with the conjugate panel are not observed.

There are only two of the carefully documented CSB serum samples which do not show a measurable response with the current conjugate panel. These serum samples have recently been retested with the entire test conjugate bank and still no response was observed. Collaborator input on these patients may be beneficial to trying to sort this out. One thought may be that the isomeric form of the TDI may be critical in some cases. This may be particularly important for workers that are exposed to greater levels of the 2,6-TDI during the manufacturing of TDI-based polyurethane foam. Using the reaction schemes designed for the 2,4-TDI panel, a comparable 2,6-panel has been prepared and will be used for parallel screening of the serum samples as well. Correlation of exposure, clinical and reaction data will also be completed.

Table 10: Positive Conjugate Panel Reaction Summary

Conjugate Parameter	Conjugate 30	Conjugate 44	Conjugate 45	Conjugate 46	Conjugate 61	Conjugate 62
Protein Conc.	5 mg/ml	5 mg/ml	5 mg/ml	10 mg/ml	5 mg/ml	5 mg/ml
Reaction Volume	5 ml	10 ml	10 ml	10 ml	1 ml x 8 wells	1 ml x 8 wells
pH	9.4	9.2	7.4	7.4	9.4	7.4
Buffer	50 mM sodium borate	8% $NaHCO_3$	50 mM sodium phosphate	PBS	50 mM sodium borate	PBS
Molar Ratio TDI:HSA	250:1	400:1	200:1	700:1	0.8 ppm	0.8 ppm
Solvent	acetone	neet	acetone	neet	NA	NA
Reaction Temp.	Room Temp. (RT)	RT	0°C	RT	RT	RT
Reaction Time	1 hr	1 hr	30 min.	20 min.	1 hr	1 hr
Stirring	y	y	y	y	n	n
Quench /Dialysis	1.2 M ammonium carbonate & dialyze vs. water	dialyze vs. PBS & filter	5000 x g 20' & dialyze	5000 x g 20' &dialyze 20% TCA 5000 x g 1N NaOH dialyze	1.2 M ammonium carbonate & dialyze vs. water	1.2 M ammonium carbonate & dialyze vs. water

DISCUSSION

Biological monitoring for markers of chemical exposure and biological effect has had a significant impact on diagnosis, treatment and prevention of chemically-induced disease. It is a primary goal of clinicians and research scientists alike, to find sensitive, predictive and reliable biological markers, as well as, methods to detect such indicators both, qualitatively and quantitatively. For isocyanate compounds, finding reliable markers and methods for monitoring exposure and/or effect has been particularly difficult. This may possibly be due to the high reactivity of these compounds (1) and the complexity of the associated response processes (2). Some groups have developed biological monitoring methods which involve the hydrolytic treatment of biological samples and subsequent analysis of the corresponding diamine (3, 4). While there is a correlation with exposure, this method often does not address the original state or source of the material and therefore, is of limited value in evaluating an individual's response process.

This project was initiated in response to the need to clarify the inconsistent and confusing results on the immunological responses to isocyanates. Clinicians and researchers alike, confirmed the need to understand this complex phenomenon. The CSB collaborative project was designed with a limited focus and is moving only one step toward understanding the mechanisms and responses involved. As of the close of phase I, all of the specific goals have been addressed and many answers and questions have surfaced. As we move on to Phase II, we hope to build on the foundation of results and interactions established thus far and to further engage the expertise of our collaborators both experimentally and intellectually as we move forward.

REFERENCES

1. Asthma in the Workplace (1993).

2. O. Vandenplas. J.L. Malo, M. Saetta. C.E. Mapp and L. Fabbri (1993) Occupational Asthma and extrinsic alveolitis due to isocyanates: current status and perspectives. *Br J Ind Med. 50:* 213-228.

3. L.C. Grammer and R. Patterson (1993) Immunologic Evaluation of Occupational Asthma. In: Asthma in the Workplace. Marcel Dekker, Inc. New York, Basel, Hong Kong, pp. 125-143.

4. B. T. Butcher, C.E. O'Neil, M.A. Reed and J.E. Salvaggio (1980) Radioallergosorbent testing of toluene diisocyanate-reactive individuals using p-tolyl isocyanate antigen. J. Allergy Clin Immunol 66: 213-216.

5. X. Baur, M. Dewair and G. Fruhmann (1984) Detection of immunologically sensitized isocyanate workers by RAST and intracutaneous skin tests. J. Allergy Clin Immunol 73:610-618.

6. R. Patterson, F.E. Hargreave, L.C. Grammer, K.E. Harris and J. Dolovich. (1987) Toluene diisocyanate respiratory reactions. Int Arch Allergy Appl Immunol 84: 93-100.

7. J.M. Danks, O. Cromwell, J.A. Buckingham, A.J. Newmann-Taylor and R.J. Davies (1981) Toluene diisocyanate induced asthma: Evaluation of antibodies in the serum of affected workers against a tolyl monoisocyanate protein conjugate. Clin Allergy 11:161-168.

8. L. C. Grammer, K.E. Harris, J.-L. Malo, A. Cartier and R. Patterson (1990) The use of an immunoassay index for antibodies against isocyanate human protein conjugates and application to human isocyanate disease *J. Allergy Clin. Immuno 86:* 94-98.

9. A. Spiazzi, P. Boccagni, P. Germano, and A. Pezzini (1991) RAST-detection of specific IgE in dipenylmethane diisocyanate exposed workers: considerations in performance of the test. *Allergy 46:* 166-172.

10. U. Wass and L. Berlin (1989) Immunologic specificity of isocyanate-induced IgE antibodies in serum from 10 sensitized workers. *J. Allergy Clin Immunol. 83*: 126-135.

11. Patterson R, Hargreave FE, Grammer LC, Harris KE, and Dolovich J (1987) Toluene diisocyanate respiratory reactions: Reassessment of the problem. *Int Arch Allergy Appl. Immunol.* **84**: 93-100.

12. R. Jin and M.H. Karol (1988) Diisocyanate antigens that detect specific antibodies in exposed workers and guinea pigs. *Chem. Res. Toxicol. 1*: 288-293.

13. Schroeder R and Moore KD (1985) A reverse phase liquid chromatographic determination of hexamethylene diisocyanate in inhalation chamber atmospheres. Mobay Toxicology Report, No. 639.

14. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein Measurement with the Folin Phenol Reagent. *J. Biol. Chem.* **193**: 265-275.

15. Snyder, S.L. and Sobocinski, P.Z. (1975) TNBS Determination of Amino Group Concentration. *Anal. Bioch.* **64**: 284-288.

16. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**: 680-685.

17. Robertson, E.F., Danelly, H.K., Malloy, P.J. and Reaves, H.C. (1987) Rapid Isoelectric Focusing in a Vertical Polyacrylamide Minigel System. *Anal Biochem.* **167**: 290-294.

18. Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic Transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *PNAS* **76**:4350-4354.

19. Engrall, E and Perlman, P. (1971) Enzyme-linked immunosorbent Assay (ELISA) Quantitative Asssessment of Immunoglobulin. *Immunochem.* **8**: 871-879.

APPENDIX I

MEDICAL DATA DOCUMENT
CSB SERUM SAMPLE

CSB ID#:_____

Supplying Collaborator:_____

Sample Identification: _____

Please answer as many questions as possible. If information on a specific question is not available, go on to the next question.

I. PATIENT IDENTIFICATION

1. Study Number _____
2. Employee Number _____
3. Name (optional) _____
4. Initials _____
5. Current Occupation or Job Title_____
6. Smoker _____Ex-Smoker _____Non-Smoker_____

II. EXPOSURE HISTORY

Job Titles (Most recent first) Dates

1.
2.
3.
4.
5.
6.
7.
8.

For the following questions, please check all that apply.

To which isocyanates has the patient been exposed?

TDI_____ MDI_____ HDI_____ Others_____ (Specify)
Most Recent Exposure (Date):

_____ _____ _____

Is there any industrial hygiene monitoring data of exposure to isocyanates for this person or for their job title during the years they worked with a particular isocyanate?

Yes_____ No_____

If Yes, please list the Time Weighted Average (TWA) exposures and/or the peak exposure data for the patient by year of exposure and/or job title.

_____

_____

What were the routes of exposure?

Skin_____ Inhalation_____

	TDI	MDI	HDI
Frequency of Skin Exposures			
<5 ppb	_____	_____	_____
6 - 20	_____	_____	_____
>20	_____	_____	_____

	TDI	MDI	HDI
Frequency of Inhalation Overexposures			
<5 ppb	_____	_____	_____
6 - 20	_____	_____	_____
>20	_____	_____	_____

Has the patient had any serious accidents with overexposures?

Yes_____ No_____

Please describe: _____

_____

_____

III. CLINICAL EVALUATION

Has the patient been diagnosed with isocyanate-induced

	Yes	No
Asthma	_____	____
Hypersensitivity pneumonitis	_____	____
Bronchitis	_____	____
Contact dermatitis	_____	____
Allergic rhinitis	_____	____
Lung function decrement (please specify)	_____	____

What tests has the patient had to support the diagnosis? Check all that apply and provide the specific results if available.

	Which Antigens were used in thetesting? Check all that apply.			
	TDI	MDI	HDI	Other Isocyanates
RAST	___	___	___	_____
ELISA	___	___	___	_____
Skin Testing	___	___	___	_____
Specific Inhalation Challenge Testing (Give concentration and duration of exposure)	___	___	___	_____
	_____			
Non-specific Inhalation Challenge Testing (Give challenge agent, concentration, and duration of exposure)	_____			
Spirometry (Indicate Test)	_____			
Peak Expiratory Flow Rate Testing	During work: _____			
	At home: _____			
Other Testing	_____			
	_____			

Has the patient's blood been tested for:

	Yes	No	Results Positive	Negative
HIV Antibody	___	___	___	___
Hepatitis C Antibody	___	___	___	___
Hepatitis B Surface Antigen	___	___	___	___

IV. OTHER RELEVANT MEDICAL HISTORY

Is there any other relevant medical information you think is pertinent to the evaluation of this patient's blood as it relates to isocyanate-protein conjugates?

APPENDIX II

CSB Participant Conjugate Questionnaire

Please complete the following information for your test conjugate.

I. Laboratory Identification:

1. Principal investigator_____
2. Address_____
   _____
3. Phone number_____
4. FAX number_____
5. Date of submission to bank_____
6. Is this your first interaction with the bank?_____

II. Conjugate: General Information:

1. Conjugate ID number_____
2. Study number_____
3. Date of preparation_____
4. Carrier protein_____
   a. Source_____
   b. Catalogue number_____
   c. Lot number_____
   d. Purity (globulin %)_____
   e. Buffer /pH_____
   f. Protein concentration_____

III. Conjugate: Isocyanate Modification:

a. Isocyanate_____
b. Supplier_____
c. Lot number_____
d. N=C=O Purity_____
e. N=C=O Storage Conditions_____
f. Method of administration_____
g. Pipetting device (glass, plastic,etc.) _____
h. Delivering Solvent_____
f. Mixing method_____
g. Molar ratio (NCO/protein)_____
h. Reaction vessel (glass, plastic, etc.)_____
i. Reaction volume_____
j. Reaction temperature_____
k. Method of isocyanate quantitation_____

IV. Conjugate: Purification

a. Quenching reagent_____
b. Filtration method_____
c. Precipitation method_____
d. Dialysis?_____
e. Lyophilization?_____

V. Conjugate: Biochemical Characterization

a. TNBS assay_____
   % modification determined_____
b. Absorption spectra_____
   % modification determined_____
c. Other methods_____
   % modification determined_____
d. Electrophoretic analysis_____

VI. Conjugate: Immunological Characterization

a. Primary Antisera information_____
   _____
b. Secondary Antibody information_____
   _____
c. Detection system_____
d. ELISA?_____
   titer?_____
e. RAST?_____
f. Dot blot?_____
g. Western blot?_____
   M.W. reactive bands?_____

VII. Additional Information/Comments:

APPENDIX III

The Bank

The Newsletter for CSB Participants

Conjugate Serum Bank
*A Collaborative Project**

Volume 1 *September, 1994* *Number 1*

Director's Notes...

• Last August, the Conjugate Serum Bank (CSB) Project was initiated. The first goal was to establish a nucleus of collaborators, primarily consisting of participants of the Warren House Workshop on Pulmonary Sensitization By Isocyanates. It was at this meeting, the concept of a centralized bank was discussed and supported. Nine laboratories were invited to participate. To date, seven groups have agreed to take part in this collaborative endeavor. See Page 2 for Directory Listings.

• In order to coordinate the CSB project, a working Task Force has been established under the auspices of the International Isocyanate Institute. The Task Force Directory is given on Page 2. An update from the current chairman is given on Page 3.

• Over 80, variable parameter, TDI-protein conjugates have been made as part of the first phase of the CSB project. These conjugates have been biochemically characterized and tested. See Conjugate Characterization section for details.

• Forty-six serum samples from 4 CSB participant laboratories have been collected. These serum samples, from TDI-exposed individuals, have been used to screen for conjugate recognition. Medical data has been recorded for each sample and is now being used to try to make correlations. See Sera Screening section for data summary.

• The initial phase of the CSB project is nearly complete. It is now time to review our objectives, evaluate our status and to examine our future directions. One of the advantages of this project is that we all have the opportunity to draw upon the expertise of a number of leading laboratories to address a common problem. As a CSB participant, it is critical to have your input and advice. Please complete and return the enclosed response card and by doing so, help to mold this project from your perspective.

The CSB Project

Isocyanate-induced asthma is a complex entity which has been difficult to understand, diagnose and treat. While there are numerous etiologies to investigate, the initial phase of the CSB project was designed to systematically characterize the effects of test antigen preparation and testing. Many laboratories have attributed some of the diagnosis difficulties to conjugate preparation. Through this collaborative endeavor we hope to address this issue and provide a foundation of information upon which we can all build.

The Objective:

The initial project objective is to develop standardized methods for diisocyanate conjugate preparation and sera testing. An important goal of the project is to establish a centralized bank of well characterized conjugates and antisera. In doing so we hope that this collaborative research endeavor will bring together the expertise of biochemists, immunologists, toxicologists and clinicians to improve the testing, diagnosis, treatment, and prevention of isocyanate-induced asthma.

* Complete list of current participants in collaborative project on page 2.

Inside...

Conjugate Characterization 3
Future Directions and Participant Survey.....4
Participant Directory.....2
Sera Screening .....3 & 4
III Task Force Directory.....2

Current CSB Participant Directory

Participant Laboratory	Address	Phone/FAX
Dr. X. Baur	Berufsgenossenchaftliches Forschungs Institut fur Arbeitsmedizin (BGFA) Gilsingstrasse 14 D-44789 Bochum Germany	PH: 49-234-302-6393 FAX: 49-234-302-6372
Dr. I.L. Bernstein	University of Cincinnati Medical Center 231 Bethesda Ave. ML#563 Cincinnati, OH 45267 USA	PH: 513-558-4701 FAX: 513-558-3799
Dr. M. Karol	Dep't of Environmental and Occupational Health University of Pittsburgh Graduate School of Public Health 260 Kappa Dr. Pittsburgh, PA 15238 USA	PH: 412-967-6530 FAX: 412-967-6611
Dr. A. L. Kennedy /Dr. W. E. Brown	Carnegie Mellon University Dep't of Biological Sciences 4400 Fifth Ave. Pittsburgh, PA 15213 USA	PH: 412-268-3186 FAX: 412-268-7129
Dr. S. Kochman	Centre Hospitalier Universetaire 45,Rue Cognacq Jay F-51100 Reims, France	PH: 33-26-78-76-17 FAX: 33-26-05-87-06
Dr. J.-L. Malo	Hopital Du Sacre'-Coeur De Montreal Service de pneumologie-recherche 5400, boulevard Goiun Ouest Mor.treal (Quebec) Canada H4J1C5	PH: 514-338-2796 FAX: 514-338-3123
Dr. C. Mapp	Istituto Medicina del Lavoro Universita degli Studi di Padova Via J Facciolati 71 35127 Padova Italy	PH: 39-49-821-6627 FAX: 39-49-821-6631

CSB Project
International Isocyanate Institute Task Force

Task Force Member	Address	Phone/FAX
Dr. Dennis Allport	Gilbert International Isocyanates* The Scientific Office, P.O. Box 42, Hexagon House Blackley, Manchester M9 8ZS UK * Gilbert International Isocyanates, an independent contractor, operates the Scientific Office of III	PH: 44-61-721-1575 FAX: 44-61-721-2395
Dr. Athena Jolly	850 Penns Way West Chester, PA 19382	PH: 610-447-2898 FAX: 610-793-2411
Dr. Timothy Landry (chair)	DOW Chemical Co. 1803 Building Midland, MI 48674	PH: 517-636-2733 FAX: 517-638-9863

Task Force Update...

• The Warren House conferees advocated the establishment of a centralized bank for diisocyanate serum conjugates; many have provided serum samples for this effort. We appreciate the support of CSB participants. These investigators have a great deal of expertise on diisocyanates and occupational asthma, and we hope that the CSB will provide a mechanism for expanding the science in this area. A CSB meeting is planned concurrent with the American Thoracic Society meeting in Seattle (May, 1995). Although CSB travel funds are not available, we hope that this will be a convenient venue for many scientists who are interested in hearing about our progress and helping to determine our future direction. More information will be forthcoming, though we welcome your comments on this or any CSB activity.

Kind Regards,

Tim Landry, Ph.D.

Conjugates:

Preparation and Characterization...

The role of test conjugate preparation in relation to the difficulties in detection of isocyanate antibodies has often been postulated in the literature but has not been fully characterized. Based on results of *in vitro* studies performed in our laboratory as well as protocols given in the literature, we began the synthesis of several variable parameter, TDI-protein conjugates. Some of the parameters altered included: reaction pH, reactant concentrations, mode of isocyanate addition, time of reaction, and use of quenching agents.

Initial biochemical characterization of each test conjugate was performed. Amino and sulfhydryl group modification was quantitated by the TNBS and Ellman's assays. Intra and intermolecular crosslinking was assessed using SDS Polyacrylamide gel electrophoresis. Isoelectric variations were monitored through IEF PAGE. Extent of modification was also analyzed through spectrophotometric scanning. Reproducibility of conjugate preparation methods is being monitored as well.

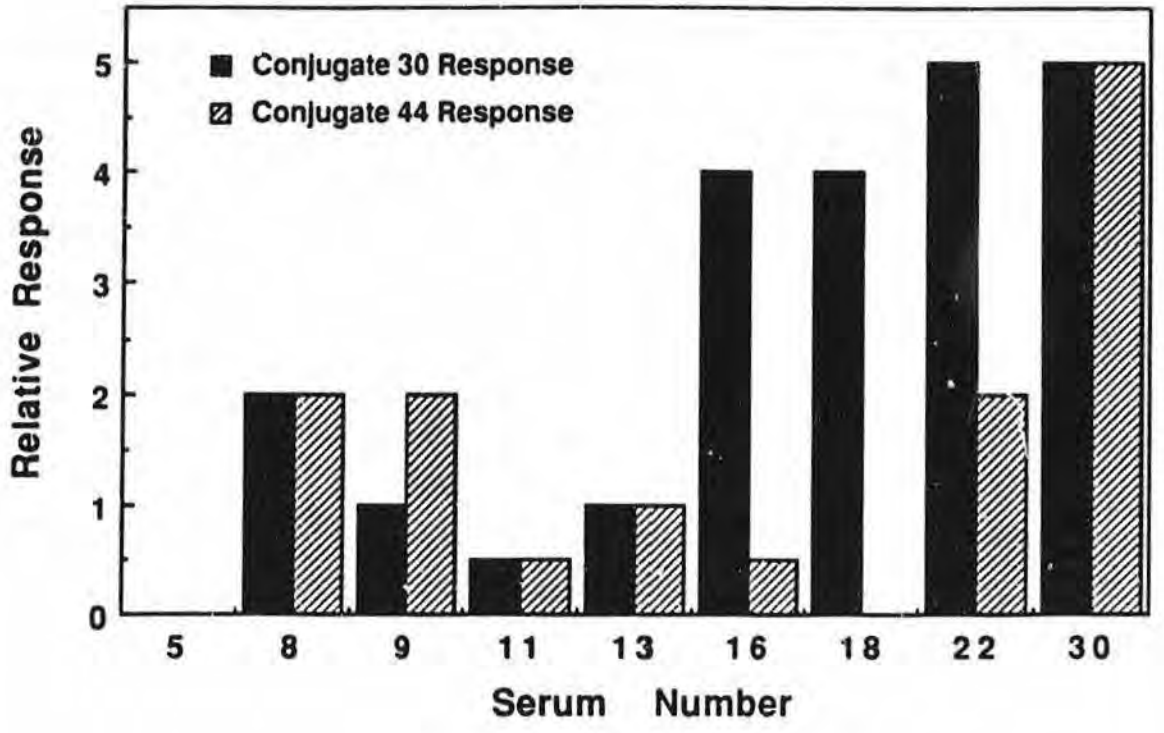
Aliquots of characterized conjugates were then used as test antigens to screen the serum bank. Conjugates which have shown positive reaction toward a number of bank serum samples are being mapped in detail to determine molecular reaction sites.

Serum Sample Screening:

The 46 serum samples from TDI-exposed individuals which have been received have been logged into the bank and have been used in initial dot blot screening. This immunodetection method uses the same reaction principles as ELISA but minimizes the amount of test conjugate and serum sample used.

Initially, a single, well-characterized serum sample was used to screen the conjugate bank. Four conjugates were selected based on their reactivity and were defined as the initial conjugate panel for screening the remainder of the serum samples. Screening of the TDI conjugate panel has demonstrated sera specific reaction responses. With the exception of a single bank serum, all samples showed positive response with one or more conjugate. Reaction variability of each serum sample with the conjugate panel has been observed The table below gives representative response profiles for the two most reactive conjugates against a variety of serum samples. The screening results support the hypothesis that a panel of conjugates is required, in some cases, to permit detection of positive sera. With the conjugate and sera banks narrowed, quantitative analysis through the use of ELISA testing is now being performed.

Representative Response Profiles
CSB Sera Screen



As a result of the initial phase of the project, a potential TDI conjugate panel has been developed and characterized. Once the first phase is completed, we plan to produce larger quantities of the conjugate panel. These samples would be available for distribution to collaborators who may be interested in testing them and providing feedback. We would also like to begin similar studies for MDI as well. Many groups have indicated that they could supply MDI serum samples. If you are willing to do so, please indicate this on the enclosed response card.

Now that the project is off the ground, it is critical that the participants begin to take on a more active role. For example, it would be useful to have your input on conjugate preparation procedures and detection methods which are particularly successful in your laboratory. Please provide any procedures or troubleshooting pointers which you have discovered. The compiled results will be reported to all CSB participants and hopefully included in a collaborative publication on the project.

We do also realize that an immune mechanism is only one of the many possible etiologies of isocyanate-induced asthma. We would therefore appreciate your input on other markers which could be characterized in the bank samples in future phases and your potential involvement.

Finally, for the next newsletter, we would like to include a reference listing. We hope this will be useful for all collaborators to keep up to date on the findings from each group. Please forward recent reference citations from your group to be included in the listing.

Thank you for your participation.

Sincerely,

Amy L. Kennedy, Ph.D.
CSB Project Director

CSB Participant Response Card
Newsletter 1.1

CSB Participant: _____

Please indicate your response(s) and return.

A. Serum samples from MDI-exposed Individuals

My group has serum samples from MDI-exposed individuals which we would be willing to supply to the bank for conjugate screening.

Approximate number of samples: _____
Estimated date of shipment: _____

B. Conjugate Preparation and Sera Screening Methods Survey

My group has found the following methods for conjugate preparation and screening particularly useful/critical: (Please provide reference citations, when possible.)

Preparation: _____
_____
_____

Screening: _____
_____
_____

Specific Key Pointers: _____
_____
_____

C. Future Directions for the CSB Project

I would like to see the project move forward in the following direction(s):

_____
_____
_____

D. Reference Citations

My group has recently published the following papers on isocyanates or related topics which may be of interest to other participants.

_____
_____
_____

E. CSB Participant Meeting Interest

____ My group will be represented at the American Thoracic Society Meeting in Seattle in May, 1995 and would be interested in having a CSB meeting there.

____ My group will not be represented at the ATS meeting and I would propose to meet in conjunction with the following meeting, instead: _____

____ I am not interested in participating in a CSB meeting at this time.

F. Additional Comments/Suggestions:

_____
_____
_____

(fold here)

Dr. Amy Kennedy
Conjugate and Serum Bank Project
Department of Biological Sciences
Carnegie Mellon University
4400 Fifth Avenue
Pittsburgh, PA 15213

AMTEK CORP.

CERTIFICATE OF AUTHENTICITY

THIS IS TO CERTIFY that the microimages appearing on this microfiche are accurate and complete reproductions of the records of U.S. Environmental Protection Agency documents as delivered in the regular course of business for microfilming

Data produced 4 3 97 Marcia Tubolino

(Month) (Day) (Year) Camera Operator

Place Syracuse New York

(City) (State)